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The goal of this project is to improve the detection and treatment of breast cancer by characterizing vasopressin gene expression in these cells and determining the nature and role of products generated through this expression. Our earlier data suggest that vasopressin gene-related products (VGRPs) are features common to all breast cancers, and since the vasopressin gene is not apparently expressed in normal breast tissues or in benign breast disease, its expression is probably part of the carcinogenic process. Studies are now being conducted on carcinoma in situ to ascertain if expression of this gene can be used as a predictor of breast cancer in patients. RT-PCR and sequencing data indicate that breast cancer cells produce a normal VPmRNA and an abnormal VPmRNA that is extended at the 5'end. These VPmRNAs lead to the formation of 40 KDa and 20 KDa proteins, one or both of which become components of the plasma membrane and can be targeted with Abs. RT-PCR studies have also shown that breast cancer cells appear to express at least two key processing enzymes (CPE and PC2), and all four receptors to vasopressin, as well as receptors to oxytocin. Vasopressin has been shown by us to induce calcium mobilization, cytoskeleton changes, and phosphorylation of MAPK, in breast cancer cells. These latter findings provide evidence for the autocrine growth activities of vasopressin on breast cancer cells. Planned studies are designed to fully-characterize VGRPs and their regulation, and to ascertain the effectiveness of our Abs to target breast cancer in vivo.

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(5) Introduction

The overall objective of this project is to improve the detection and treatment of breast cancer by evaluating vasopressin gene-related products as tumor marker substances in hyperplastic breast disease, by characterizing the nature and regulation of the vasopressin gene and its products in breast cancer, and by determining the potential usefulness of vasopressin gene-related products on tumor membranes as targets for immunotherapy. It seeks to test the hypothesis that all breast tumors produce vasopressin as an autocrine growth factor, in situ, and that this property can be effectively utilized not only to elucidate the pathobiology of this cancer, but also to identify precancerous tissue and develop more successful treatments.

In hypothalamic neurons, vasopressin gene expression leads to the formation of a 750 bp mRNA and the subsequent generation of a 20 KDa precursor that undergoes intragranular enzymatic processing to form vasopressin (VP), vasopressin-associated human neurophysin (VP-HNP), and vasopressin-associated glycopeptide (VAG). All three of these products are released into the circulation by exocytosis. **None** of these products become components of the plasma membrane of neurons.

We have shown that the **vasopressin gene** of chromosome 20 appears to be **expressed by all breast tumors, but not by normal breast tissue** (North et al., 1995). This indicates that in the mammary gland, the expression of the vasopressin gene is a feature unique to tumor cells, a feature common to all hyperplastic tissues, or a feature shared only by tumor cells and their progenitors. The first and third of these possibilities raised the potential use of this expression as a marker of carcinogenesis, and/or forecaster of imminent disease.

Last year we reported data that strongly suggest **the vasopressin gene is an oncogenic marker of breast cancer.** In studies now submitted for publication (BJC, see Appendix), an immunohistochemical examination, using antibodies to vasopressin and vasopressin-associated glycopeptide, was performed on formalin-fixed biopsy specimens taken from 17 patients with various forms of benign breast disease, who were seen at the Dartmouth Hitchcock Medical Center between 1975 and 1984. These specimens included 4 cases of atypical ductal hyperplasia, 2 cases of fibrocystic disease with papilloma, 6 cases of fibrocystic disease with intraductal hyperplasia, 1 case with bilateral mammary hyperplasia, and 4 cases of typical fibrocystic disease. For all of these cases of benign breast disease, staining was negative for both vasopressin and vasopressin-associated glycopeptide. At the completion of the study it was discovered that three of the individuals with benign breast disease

went on to develop breast cancer. Taken together, these findings indicate vasopressin gene expression is not a marker of cellular proliferation in the breast, nor a marker of cancer progenitor cells in benign breast disease. This leads us to the conclusion that vasopressin gene expression in the breast is likely to be solely associated with the process of carcinogenesis. We are now investigating the occurrence of vasopressin gene expression in cases of carcinoma in situ (see Body of this report).

Expression of the vasopressin gene in breast cancer leads to the formation of unique gene-related products, some of which become associated with the plasma membrane of tumor cells. Because these membrane-associated products react with antibodies raised against human vasopressin-associated glycopeptide (VAG), we have referred to them as **GRSA** (Glycopeptide Related cell Surface Antigen). Because they are located at the cell membrane of the tumor cells, we have demonstrated they can be targeted, in *vitro* , with antibodies to VAG. This raises the possibility they can be utilized for targeting tumors in patients through immunotherapy. We have excellent indirect evidence that strengthens this possibility. Breast cancer uniquely shares the feature of membrane expression of vasopressin gene-related products with small-cell carcinoma of the lung (SCCL) , and we have shown we can successfully target these products in SCCL patients using radioiodinated and Indium-labelled antibodies (North et. al, 1989, North).

What is the nature of GRSA? The VPmRNA and protein products that arise in breast cancer through expression of the vasopressin gene appear to be both structurally normal and abnormal (see Body of this report). We had anticipated this possibility because we (and others) have earlier shown that abnormal and normal forms co-exist in SCCL (North et. al, 1983; Rosenbaum et. al, 1990; North and Yu, 1993). There appear to be two VPmRNAs in both breast cancer and SCCL. One of these is sequentially almost identical to that in human hypothalamic neurons, while the other is extended by 600 base pairs at the 5' end of the reading frame. The VPmRNAs of both types of tumors give rise to proteins of 40 KDa and 20 KDa as prominent forms, although the proteins of breast cancer appear to show some structural differences to those of SCCL (North et al., 1995). The 20 KDa form of SCCL is almost identical to the provasopressin of hypothalamic neurons. Both 40 KDa and 20 KDa proteins of SCCL become incorporated into the cell membrane as cell-surface antigens. Studies are underway to determine the structures of the two VPmRNAs of breast cancer and discover if both 40 KDa and 20 KDa proteins of this tumor type represent **GRSA** at tumor cell surfaces.

In normal hypothalamic neurons, 20 KDa provasopressin is processed by proteolysis that is thought to involve at least four enzymes. That such

proteolysis also occurs in breast cancer is evidenced by our recent preliminary findings (see progress report, 1995) that most patients with breast cancer have inappropriately high plasma levels of vasopressin, and elevated levels of VAG (unpublished data). Breast cancer can therefore be classified as neuroendocrine in nature. Because of this, we have commenced studies to examine the presence, or otherwise, of the key processing enzymes, carboxypeptidase E, and prohormone convertases PC2 and PC1/3, in the two breast cancer cell lines MCF7 and ZR-75-1.

Vasopressin can act as a growth factor/growth modulating agent, and in SCCL lines it promotes calcium mobilization and clonal growth (Hong and Moody, 1991; Sethi and Rozengurt, 1991). Last year we reported that vasopressin can promote calcium mobilization in two breast cancer cell lines, ZR-75-1 and T47D, and can dramatically influence the cytoskeleton of ZR-75-1. These finding are supported by previous studies on a dimethylbenzathrene-induced rat mammary tumor (Monaco et al., 1978; Monaco et al., 1980; Guilon et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988), human MCF7 breast cancer cells (Taylor et al., 1990), and on another breast cancer cell line (Bunn et al., 1992). Choi et al. (1994) were also able to show that vasopressin promotes growth of mammary tumors in transgenic mice. These actions of vasopressin have prompted us to investigate the nature of vasopressin receptors on breast cancer cells. Four vasopressin receptors have been indentified in other cells and have been cloned (Birnbaumer et al., 1992; Hirasawa et al., 1994; Sugimoto et al., 1994; Thibonnier et al., 1994; Burnatowska-Hledin et al., 1995). These are known as vasopressin V_{1A}, V_{1B}, and V_2, receptors plus vasopressin-activated calcium-mobilizing receptor (VACM1). Although an investigation of vasopressin receptors and the growth promotional activities of vasopressin may seem to fall outside of intentions enunciated in the original proposal, we believe they nevertheless address the body of the hypothesis advanced in the proposal and fall within the goals of Technical objectives 2 and 3. It is believed that such an investigation could not only explain the seemingly universal expression of the vasopressin gene in breast tumors, but also lead to an additional number of effective therapies.

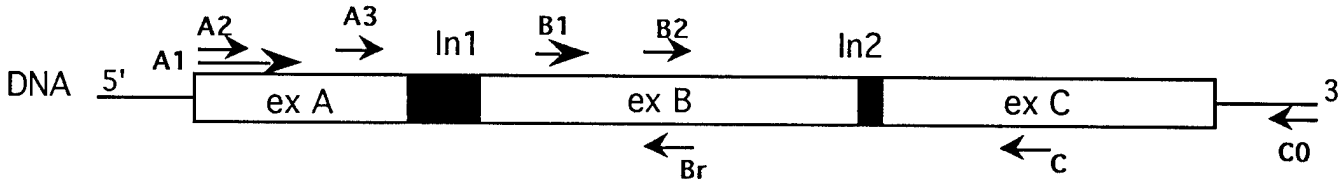
(6) Body

Technical Objective 1: Vasopressin gene-expression in breast hyperplasia as a predictor of cancer (Task 1 in Statement of Work).

As initially discussed in the previous progress report, we have now completed a survey of the incidence of vasopressin gene expression in fibrocystic disease,

and this work has been submitted for publication to the British Journal of Cancer. This immunohistochemical survey involved a blind study of archival material representing various benign breast lesions, and employed antibodies directed against two regions of the vasopressin precursor, namely the vasopressin and vasopressin-associated glycopeptide (VAG) moieties. Followups were performed on patient records to discover if they later developed breast cancer (see appendix for preprint). The outcome of our survey, combined with earlier findings from a study on breast cancer lead us to the conclusion that vasopressin gene expression is not a marker of cellular proliferation in the breast, is not a marker of cancer progenitor cells in benign breast disease, and, is therefore likely to be associated with the process of carcinogenesis in the breast. However, our argument for an absence of vasopressin gene expression in cancer progenitor cells is at best tenuous, since we had no available independent test for characterizing such cells, and our conclusion is based solely on the later development of breast cancer in three of the patients studied. In order to more clearly answer this question, we have therefore now commenced another survey that will evaluate vasopressin gene expression in pre-metastatic breast lesions such as carcinoma in situ. Blocked out biopsy samples of twenty cases of carcinoma in situ, ten of which have been clearly identified as being of the **comedo** variety with abnormal cells and extensive necrotic areas, are now being examined immunohistochemically, first with antibodies to VAG, and then with antibodies to vasopressin. Followup studies will be conducted on the records of patients from whom biopsy samples were obtained to determine which of these subjects later developed breast cancer.

Technical Objective 2: Characterization of vasopressin gene expression by breast cancer cells.
The data discussed in this section are largely unpublished.



Structure of Human vasopressin gene and locations of some designed PCR primers

We have established for breast cancer cells that there is abnormal, in addition to normal, production of vasopressin. Abnormal protein forms constituting GRSA might to be generated from one normal and one abnormal gene. RT-PCR, cloning, and sequencing studies on messages from the vasopressin gene of MCF7, T47D, and ZR-75-1 cells have now shown that there appear to be at least two VPmRNAs expressed in breast cancer, one from a 'normal' gene and the product of normal splicing, the second either from a 'normal' gene and the product of

alternate splicing or from an abnormal gene having insertions in exon A . The ten primers used in studies conducted this year are illustrated in the Figure above and described in the following Table 1:.

TABLE 1. of forward and reverse primers designed for RT-PCR amplification of human vasopressin gene fragments from human breast cancer cells

Forward primer	Length	Nucleotides	Exon	Sequence
A1	21	269-289	1	5'-cttctcctccgcgtgctactt-3'
A2	18	269-286	1	5'-cttctcctccgcgtgcta-3'
A3	21	321-341	1	5'-atgtccgacctggagctgaga-3'
IN	21	1504-1524	intron 1	5'-gtcatccaagaaaccaaggtg-3'
B1	25	1751-1775	2	5'-tgcttcgggcccagcatctgctgcg-3'
B2	22	1830-1851	2	5'-tgccaggaggagaactacctgc-3'
Reverse primer				
INR	20	1517-1536	intron 1	5'-agatctgctcggcaccttgg-3'
Br	22	1830-1851	2	5'-gcaggtagttctcctcctggga-3'
C	22	2152-2173	3	5'-agcaacgccacgcagctggacg-3'
C0	25	2231-2255	3	5'-taggcgtcgggctgggcgggctcga-3'

Normal-sized VPmRNA fragments of 313 bp using **A3C** were obtained from three cell lines. These have been partially sequenced and shown to have a sequence very similar to the VPmRNA found in hypothalamic neurons. We also isolated, and successfully reamplified (but have not yet sequenced) an RT-PCR product(s), from all three cell lines using the specific primers **A1** and **C**, that is 600 bases larger than that predicted from the structure of VPmRNA. Such a structure could represent **a VPmRNA that have retained a 600 base portion of intron 1 through alternative splicing** (the entire intron 1 segment contains 1373 bases). If the 5' sequence of the product confirms it translates a protein with the N-terminus of provasopressin, it will offer one explanation for the 40,000 dalton species of breast cancer because an extra 600 bases represents an additional 200 amino acid residues. Adding 200 amino acid residues to the 20,000 dalton provasopressin would give a protein of 40,000 daltons. Since antibodies recognize the exon B (at least in Western analyses) and exon C regions of the protein (North et al., 1995) the intronic insertion would not apparently cause a reading frame shift. The structure of the enlarged form will now be checked through reamplification using both **A2** and **C** primers, and **A3** and **C** primers. If the additional 600 bases in **A1C** are from intron 1, we expect in all cases, reamplified products that are approximately 600 bases larger than

predicted from normal VPmRNA. However, if products of normal size are produced this will suggest the enlarged form represents **an abnormal vasopressin gene having a 600 base insertion in the exon A region.** This insertion would be **between bases corresponding to the vasopressin and neurophysin structures.** Structures **A2** and **A3** are only separated by 35 bases in normal VPmRNA. While a definitive answer regarding the enlarged form will be best provided through cloning and DNA sequencing, the planned exercise will enable us to eliminate the possibility of alternative co-existing forms. Use of primers **B1**, **B2**, **C** and **Co** will likewise enable us to discover if forms extended in the exon B and/or exon C region exist is breast cancer cells (as found by us in SCCL), while use of the forward **IN** and reverse **INR** primers will allow us, when used with **B** and **A** primers, to obtain shortened RT-PCR products for sequencing if regions of intron 1 are indeed included in the abnormal VPmRNA structure. All of these primers have already be used somewhat successfully by us in sequencing VPmRNA forms from SCCL (unpublished).

Despite the exciting prospect that sequences could soon be available for the VPmRNA form(s) that give rise to 40 KDa and 20 KDa GRSA of breast cancer, we have now initiated efforts to perform Edman sequencing on purified samples of these proteins. We have decided to concentrate our studies on protein obtained from the cell line ZR-75-1 and will use both cultured cells and tumor xenografts in nude mice as the protein sources. Purification will employ pH-salt separations, molecular sieve chromatography, and affinity chromatography on columns of Antivasopressin-Sepharose. Our antivasopressin monoclonal antibody, DEN1, has already been used to generate the affinity resin. Protein mixes from affinity chromatography will be S-alkylated and then separated. We intend separating the 20 KDa and 40 KDa protein forms using SDS-PAGE, and either eluting the them directly into dialysis sacks or performing Western transfer to PVDF, and performing solid-phase sequencing.

PCR studies on DNA preparations from breast cancer cell lines have also been conducted using a mixture of specific primers for the vasopressin gene and oxytocin genes. This is because a published study (Morris et al., 1995) has indicated that some hypothalamic neurons in rats can express protein products that are a composite of provasopressin and pro-oxytocin through a cross-over between the vasopressin and oxytocin genes on chromosome 20. We have now established that **there is no evident cross-over between the vasopressin and oxytocin genes in breast cancer.**

Studies have also been initiated to examine sub-cellular trafficking in ZR-75-1 breast cancer cells. **Sucrose-gradient sub-fractionation** of these cells (10^8 cells/batch) has already been performed and evaluation will now be conducted by Western analysis and by RIA (VP, VP-HNP, VAG). A similar study conducted

on SCCL cells revealed that approximately 80% of the 20 KDa and 40 KDa proteins are located in the plasma membrane. Of the remaining 20%, most (90%) is found outside secretory granules, and approximately 10% is within these granules. The procedures employed were found by us to preserve granules from hypothalamic neurons with >90% of vasopressin gene-related products located in the granular fraction. Hence, either the granules of SCCL are more susceptible to rupture, or only a small percentage of translated protein is potentially processed to active hormone within these granules and then secreted. This implies that packaging is limited and most protein in SCCL cells is destined for agranular targeting to the plasma membrane. Both 20 KDa and 40 KDa proteins were found in the granular fraction of SCCL cells. This indicates that the 40 KDa product shows a capacity similar to the 20 KDa product to be packaged in the Golgi apparatus of SCCL cells. The study now underway should enable us to determine if the 20 KDa and 40 KDa proteins of breast cancer cells are trafficked in a manner similar to that in SCCL. If this is the case it will imply that the limited processing of 20 KDa and 40 KDa vasopressin gene-related proteins in breast cancer is largely due to limited packaging of translated material, rather than to an absence of processing enzymes.

The breast cancer cell lines MCF7 and ZR-75-1 were examined for the expression of mRNAs for the processing enzymes carboxypeptidase E (CPE), and prohormone convertases PC2 and PC1 (or PC3) using RT-PCR, cloning, and sequencing. The primer pairs used in these studies are depicted in Table 2 below.

Table 2: Primers designed for amplification cDNA fragments of prohormone convertases(PC) and carboxypeptidase E(CPE) from breast cancer cells

Subject	Forward primer	Length	Position	Reverse primer	Length	Position
PC1/PC3	5'tacttgcaagataccaggatg3'	21	540-600	5'gatggagatggtgtagatgct3'	21	1162-1182
PC2	5'gatcctctttttacaaagcagtgg3'	24	454-477	5'ggtgagcacagtcagatgctgcat3'	24	1312-1335
CPE	5'atgggaatgaggctgttgggac3'	21	631-651	5'catggagattggcagaaagca3'	21	1015-1035

RT-PCR studies on CPE provided amplified products of the size predicted from previously published studies on anterior pituitary cells using polyA+RNA from both cell lines. These products were reamplified, cloned and sequenced to provide structures identical to those published for this enzyme. In RT-PCR studies on PC2, we have so far only been able to amplify a product using polyA+RNA from MCF7. This cDNA fragment was shown by us to have the normal base sequence of the enzyme. We subsequently investigated if mRNA for PC1/3 was expressed in MCF7 and ZR-75-1. However, RT-PCR failed to show that this mRNA was expressed in either cell line. The results of these studies show that mRNAs for at least two of the enzymes necessary for processing

provasopressin to active hormone, neurophysin, and glycopeptide, are present in some breast cancer cell lines. We intend following up our studies on these processing enzymes by determining if substrate-converting enzymatic activities are present in protein extracts from breast cancer cells.

Technical Objectives 3: Identification of factors regulating the production of GRSA by breast cancer; and
4: Determination of the binding properties for antibodies of GRSA and other vasopressin gene-products at tumor cell surfaces.

We have not yet commenced studies designed to satisfy these technical objectives, and expect they will largely occupy our efforts during the last eighteen months of this award (Years 3 and 4). We have already performed studies on the regulation of vasopressin gene-expression in SCCL as part of another ongoing project, so all of the methods are at hand to enable us to proceed without pause. We are currently generating xenografts of the breast cancer cell line ZR-75-1 in nude mice in an effort to generate GRSA proteins for sequencing (see above), and this is expected to serve as a springboard for examining binding of VAG antibodies to breast tumors in vivo. Determination of the protein sequences of GRSA proteins is also expected to lead to production of specific antibodies against unique sequences in these proteins for targeting.

Vasopressin and oxytocin receptors are present in breast cancer cell lines:

Using RT-PCR and primers specific for mRNAs representing vasopressin V_{1A}, V_{1B}, and V_2 receptors, plus the recently cloned vasopressin-activated calcium mobilizing receptor (VACM1), and the oxytocin (OT) receptor (see Table 3 below), we have initiated an investigation of the expression of these mRNAs using polyA$^+$RNA from the five cultured breast cancer cells, BT 549, MCF7, MDA-MB-231, T47D, ZR-75. These studies represent **unpublished data.** Products of sizes predicted from normal structures and representing mRNAs for all of receptors were able to be amplified from these breast cancer cell lines. Sequencing of the V_{1B} product from MCF7 cells verified that this had sequence identity to the human V_{1B} receptor mRNA from pituitary cells. The sequencing of the product from MCF7 cells showed it to have a high degree of homology with the published rabbit VACM sequence. This represents the first demonstration of the existence of human VACM1 and the first sequence data obtained for this human mRNA. These studies therefore provide strong support for the present of all four vasopressin receptors, and of the oxytocin receptor, in breast cancer cells.

TABLE 3. Forward and reverse primers used to study the expression of mRNAs for vasopressin and oxytocin receptors.

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a. Oxytocin receptor primers
forward primer 5'-CCTTCATCGTGTGGCTGGACG-3'
reverse primer 5'-CTAGGAGCAGAGCACTTATG-3'
The oxytocin receptor primer pair amplify a PCR product of 391 bp

b. Vasopressin V_{1A} receptor primers
forward primer (bp 939-958) 5'-TGTGTCAGCAGCGTGAAGTC-3'
reverse primer (bp 1325-1346) 5'-GGACTTGGAAGATTTAGGCGAG-3'
The V1A primers amplify a 408 bp PCR product.

c. Vasopressin V_{1B} receptor primers.
forward primer (bp 1154-1173) 5'-CCAATGTGGCTTTCACCATC-3'
reverse primer (bp 1372-1392) 5'-TAGGCTGAGGCTGAGGCTGAG-3'
the V1b primers amplify a PCR product of 313 bp.

d. Vasopressin-activated calcium mobilizing receptor primers.
forward primer (bp 1825-1848) 5'-GAATGGCTAAGAAGAGAAGTTGGTATG-3'
reverse primer (bp 2475-2498) 5'-TCTTCTCTCATCCTTTCTGTAGTG-3'
the VACM primer pair amplify a PCR product of 674 bp.

e. VACM receptor primers.
forward primer (bp 1679-1702) 5'-CACCATTAAGCAAAACTACCTCTG-3'
reverse primer (bp 1802-1825) 5'-CATACCAACTTCTCTTAGCCACTC-3'
This VACM1 primer pair amplify a PCR product of 193 bp.

Northern Blot analysis of VACM receptor in breast cancer cells.

This is unpublished data. Total RNA from ZR-75-1, MCF7 and T47D breast cancer cells was examined for the present of mRNA for VACM1 vasopressin receptor by Northern analysis using a 674 bp cDNA product from RT-PCR (see above). The results obtained show that this receptor mRNA is present in all three breast cancer cell lines and represented by three distinct bands at 3.5, 5, and 6.5 Kb. At this stage it is unclear if these multiple bands include precursor mRNAs and degradation product, or multiple isoforms. As an auxilliary project the laboratory is currently attempting to clone the human VACM receptor. These results are being presented as part of a poster at the 1996 annual meeting of the American Society for Cell Biology (See abstract in appendix).

Neuropeptide-induced tyrosine phosphorylation of mitogen-activated protein kinase in MCF7 cells.

These results are not published. Taylor et al.(1990) have previously demonstrated that vasopressin is capable of stimulating the growth of MCF7 cells in vitro, and Choi et al.(1994) have shown that vasopressin stimulates breast cancer growth in vivo. However, the mechanism through which vasopressin is exerting this effect, and the receptors involved, remain uncharacterized. One common pathway for growth factor action is stimulation of the mitogen activated protein kinase (MAPK) cascade. Activation of MAP kinase occurs through phosphorylation by MAPKK of a tyrosine, then a threonine, on MAPK. Using commercial antibodies against phosphotyrosyl MAPK and Western analysis, we have commenced an investigation of vasopressin activation of MAPK in MCF7 cells. Our results clearly show that vasopressin, and a specific vasopressin V1 agonist, induce an significant increase in phosphotyrosyl MAPK. They therefore indicate that vasopressin is inducing signaling events in breast cancer cells that could lead to cancer cell growth.

Ion currents in T47D human breast cancer cells.

Although not directly related to this project, we have initiated a collaborative project with Dr. Frances McCann of this school to identify and characterize ion currents in breast cancer cells, since ion signaling events have been linked to mitogenesis (Dubois and Rouzaire-Dubois, 1993). Using the whole cell configuration of the patch-clamp technique we have identified a chloride current and a voltage- potassium current in T47D human breast cancer cells. For details of these findings see the reprint in the appendix (Gallagher et al., 1996). We are now examining if vasopressin induces ion currents in these breast cancer cells.

(7) CONCLUSIONS

The original hypothesis advanced for this project proposed that all breast tumors produce vasopressin an an autocrine growth factor, in situ, and that this property can be effectively utilized not only to elucidate the origin and pathobiology of this cancer, but also for identifying precancerous tissue and developing more successful treatments. All of the studies contained in this report have provided further evidence supporting and strengthening most aspects of this hypothesis. The one aspect not supported by our data is that dealing with possible the predictive nature of vasopressin gene expression by precancerous lesions. This is from a study conducted by us on benign fibrocystic breast diseases demonstrating an absence of vasopressin gene expression even in three cases where breast cancer later developed. While non-supportive, the evidence is at best tenuous because there is no confirmation except outcome that

precancerous lesions were present in the biopsy samples examined. A much clearer picture of the possible predictive nature of vasopressin gene expression is expected to come from our current immunohistochemical study on carcinoma in situ.

Further confirmation has been obtained that the vasopressin gene is a universal marker of breast cancer and that this expression is part of the carcinogenic process in breast. This makes possible the application of methods for evaluating this expression in biopsied tissue to be used for effective screening. It would also appear that breast cancer can be generally defined as a neuroendocrine tumor because of our finding that some products of the vasopressin gene are elevated in the plasma of patients. Confirmation of these data may provide an alternate plasma procedure for diagnosing breast cancer and for monitoring treatment. Plasma RIAs for vasopressin gene-related products have already proven useful for patients with small-cell lung cancer (North, 1991).

We have now determined that in breast cancer cells there exist both normal and abnormal VPmRNAs. The normal VPmRNA is believed to lead to the production of 20 KDa GRSA, most probably a glycosylated provasopressin very similar to the provasopressin of human hypothalamic neurons. RT-PCR and sequencing have revealed a portion of the sequence of this 'normal' mRNA, and shown other portions are of the expected length. RT-PCR has also disclosed the presence of an VPmRNA that is 600 bp larger than normal VPmRNA, and these additional bases are in the 5' region of the molecule. The placement of the primers employed suggests this abnormal VPmRNA is either a product of alternate splicing that partially retains intron 1 of a normal vasopressin gene, or a product of an abnormal (mutant) vasopressin gene. The abnormal VPmRNA is thought to give rise to 40 KDa GRSA because the extra 600 bp of this mRNA should translate to an additional 20 KDa of protein product. RT-PCR and DNA sequencing using the primer pairs we have acquired, and our planned purification and Edman sequencing of GRSA proteins, is anticipated to allow us to fully characterize the vasopressin gene(s) and VPmRNAs of breast cancer, and the protein products that are generated through their expression.

It is currently unknown if one or both of the 40 KDa and 20 KDa GRSA proteins become incorporated into the plasma membrane of breast cancer cells, and this is expected to become clear from the sub-cellular trafficking study we have now initiated. Other questions that will be answered by the information from this study relate to, the nature of post-translational processing of proteins, the reason proteins are targeted to the plasma membrane, and the presence or absence of processing enzymes in secretory vesicles. Our reported RT-PCR studies on two key processing enzymes present in hypothalamic neurons have demonstrated that mRNAs for both carboxypeptidase E and prohormone

convertase 2 are expressed in breast cancer cells. These data imply that if post-translational processing is limited in breast cancer, it is not due to the absence of these enzymes.

Our hypothesis proposes that vasopressin is an autocrine growth factor for breast cancer, and all of our recent findings support this contention. RT-PCR and sequence studies have demonstrated that all four vasopressin receptor sub-types (V_{1A}, V_{1B}, V_2, and VACM1) are expressed by breast cancer cells. Our report includes the discovery and first description of human VACM1 receptor. Oxytocin receptors have also been shown by us to be present in breast cancer cells. Additionally, we have demonstrated that vasopressin and vasopressin V_1 agonists can produce in these cells, a mobilization of intracellular calcium, a change of cytoskeletal architecture, and an activation of MAP through phosphorylation of tyrosine. These are mechanisms generally recognized to be involved in cellular growth promotion and cellular mobilization.

Investigations planned over the next two years include those related to the regulation of vasopressin gene expression, and to the capacity of antibodies against vasopressin-related glycopeptide (anti-VAGs) to target breast tumors in vivo. The outcome of the first of these investigations has important implications for the second, because if clinically acceptable methods can be devised for upregulating GRSA at tumor cell surfaces, then improved targeting of breast cancer in patients by anti-VAGs should result.

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1996 STANDARD ABSTRACT FORM

EVIDENCE FOR THE EXPRESSION OF A NOVEL VASOPRESSIN -ACTIVATED CALCIUM MOBILIZING RECEPTOR (VACM-1) IN HUMAN BREAST CANCER AND LUNG CANCER ((K.A. Longo, M.J. Fay, J. Du, and W.G. North)) Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756

The purpose of this study was to determine if a human homologue of the rabbit VACM-1 receptor is expressed in human cancer cells. Research indicates that vasopressin may be involved in human breast cancer and lung cancer pathophysiology, as an autocrine/paracrine factor. Vasopressin can act through four classes of receptors: V2, V1a, V1b, and the recently cloned VACM-1, a structurally unique member of this group that contains a single transmembrane domain. Vasopressin induced an increase in intracellular free Ca2+ in the breast cancer cell lines MCF-7, T47-D, and ZR-75 as well as in the lung cancer cell line H-146. RNA from these cell lines, as well as normal human tissues (kidney and lung), was used for reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. RT-PCR, using two primer sets designed against the rabbit VACM-1 sequence, amplified bands of the predicted sizes of ~674 bp and ~193 bp in all cell lines and tissues tested. Direct sequencing of PCR products obtained from MCF-7 and H-146 revealed a high degree of identity to the cloned rabbit VACM-1 cDNA sequence. Northern blot analysis, using the 674 bp PCR product as a probe, revealed the presence of three distinct bands, of approximate sizes 3.5, 5 and 6.5 kilobases, in the cancer cell lines. In summary, we have demonstrated the presence of mRNA for a novel vasopressin receptor in human cancer cell lines and normal human tissues.

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THERE IS A $10 FEE TO REPLACE ABSTRACTS

Immunohistochemical evaluation of vasopressin gene expression in fibrocystic breast disease.

Michael J. Fay[1], Rennie S. Mathew[1], Edward M. Donnelly[1], Vincent A. Memoli[2], and William G. North[1,3]

Departments of Physiology[1] and Pathology[2], Dartmouth Medical School, Lebanon, NH 03756-0001

Summary: We previously found that expression of the vasopressin gene is a common feature of human breast cancer. In the present study we examined 17 different cases of benign fibrocystic breast disease for vasopressin expression using immunohistochemistry and antibodies directed against vasopressin and vasopressin-associated glycopeptide. All cases examined were negative for vasopressin gene expression using these antibodies. These results suggest that vasopressin gene expression occurs as part of the carcinogenic process rather than being a marker of cellular proliferation in the breast.

Key Words: Vasopressin, fibrocystic breast disease, immunohistochemistry

Running Title: Vasopressin and fibrocystic breast disease

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Introduction

Although a number of risk factors have been identified as possible causative agents for breast cancer, the etiological origin of this disease remains obscure (Henderson, 1993). Among those conditions which are predisposing towards breast cancer are proliferative breast disease, particulary atypical ductal and lobular hyperplasia (Dupont and Page, 1985; London et al., 1992; Connolly and Schnitt, 1993; Dupont et al., 1993). Atypical hyperplasia is classified as a borderline lesion because it has some of the histological features of carcinoma *in situ*. Previously we found that expression of the vasopressin gene is a common feature of human breast cancer using immunocytochemistry and antibodies directed against different regions of the vasopressin precursor (North et al., 1995). These results lead to the possibility that vasopressin expression could either, be a marker of cellular proliferation, represent part of the oncogenic process, or be a recognizable feature of cancer progenitor cells in precancerous breast lesions. We have commenced efforts to examine these questions by performing an immunohistochemical evaluation for vasopressin gene-expression using archival material representing various fibrocystic breast lesions.

Materials and Methods

Tissues

Formalin-fixed biopsy specimens were obtained from 17 patients with various forms of benign breast disease who were examined between 1975 - 1984 at Dartmouth Hitchcock Medical Center (DHMC, Lebanon, NH). The cases included, 4

cases of fibrocystic disease without hyperplasia, 9 cases of fibrocystic disease with ductal or lobular hyperplasia, and 4 cases of fibrocystic disease with atypical ductal hyperplasia. Diagnosis from pathology reports was confirmed by examining hematoxylin- and eosin- stained sections. These cases were followed for the subsequent development of breast cancer. Formalin-fixed specimens of human hypothalamus and pituitary were obtained from autopsies performed at DHMC.

Antibodies

Rabbit polyclonal antibodies directed against vasopressin and the 18 amino acid C-terminal vasopressin-associated glycopeptide were prepared using previously published methods (North et al., 1991; Friedmann et al., 1994). Antibody purification involved ammonium sulfate precipitation of the immunoglobulin fraction, and fractionation on a column of protein A Sepharose with pH gradient elution (pH 7.6 - pH 3.0). Antibodies were obtained as a pH 4.0 subfraction, dialyzed and lyophilized. Based on dilution trials, antibodies to vasopressin and vasopressin-associated glycopeptide were used at concentrations of 11 ng/ml (1:2,000) and 190 ng/ml (1:800), respectively. Protein concentrations were determined using differential spectroscopy (Waddell, 1956).

Immunohistochemistry

Sections of 4 - 6 microns from each specimen of fibrocystic breast disease were deparaffinised and stained for vasopressin and vasopressin-associated glycopeptide using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA,

USA) and avidin-biotin complex (ABC) immunohistochemistry (Guesdon et al., 1979). Tissues were rehydrated by washing with xylene, descending concentrations of ethanol, and PBS (2 x 3 min, ambient temperature). Slides were blocked with 10% normal goat serum in PBS for 20 min at ambient temperature. The blocking solution was aspirated and sections incubated overnight at 4 °C with primary antibody diluted in PBS with 1.5% goat serum. Following incubation with primary antibody the slides were washed with PBS (2 x 3 min). Goat anti-rabbit biotinylated secondary antibody diluted in PBS containing 1.5% goat serum was applied at a concentration of 20 µg/ml for 30 min. Unbound secondary antibody was removed by washing 2 x 3 min with PBS, and endogenous peroxidase activity blocked using 3% hydrogen peroxide dissolved in absolute methanol (Streefkerk, 1972). After washing with PBS (3 x 5 min) , slides were incubated with the avidin-peroxidase complex (25 µg/ml) for 30 min at ambient temperature. Slides were washed with PBS (2 x 3 min), and visualization of bound complex was achieved by adding a solution of 3,3'diaminobenzidine (0.2 mg/ml in PBS with 0.03% hydrogen peroxide) for 2 - 5 min. Tissues were then counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol, washed in xylene, and coverslipped using permount. Antibody specificity was insured by incubating negative controls with pre-immune rabbit serum fractionated, using protein A Sepharose, at pH 4.0.

Results

Positive immunohistochemical staining was obtained for vasopressin neurons in human hypothalamus and for neuronal terminals of these neurons in the posterior

pituitary with both antibody preparations (data not shown). Alternatively, negative staining was obtained with both of these antibodies in the 17 cases of benign breast disease. In several tissue sections staining of mononuclear cells was evident. Staining of sections with pre-immune rabbit serum resulted in a lack of staining. As demonstrated previously, Fig 1a demonstrates positive staining of an acetone-fixed infiltrating ductal breast cancer biopsy specimen with the vasopressin-associated glycopeptide antibody (North et al., 1995). In this section the breast cancer cells demonstrate intense staining, and the normal ducts of the breast are unstained. Figure 1b demonstrates a tissue section of fibrocystic disease with adenosis which showed no immunostaining with the antibody to vasopressin-associated glycopeptide. A case of atypical ductal hyperplasia is represented in Fig 1c which exhibited negative staining with the antibody to vasopressin . It should be noted that the nuclei of cells appear dark because the nuclear counterstain hematoxylin was used. Follow-up of the medical records revealed that three individuals, one from each classification group, subsequently developed breast cancer.

Discussion

There is an increasing body of evidence which supports a connection between vasopressin and breast cancer. There are two published clinical reports of patients with breast cancer presenting with the syndrome of inappropriate antidiuretic hormone secretion (Gupta et al., 1986; Howard et al., 1993). Both *in vitro* and in *vivo* studies support a connection between vasopressin and breast cancer. Several studies indicate that a cell line derived from a dimethylbenz(a)anthracene-induced rat

mammary tumor possesses functional V_{1a} vasopressin receptors (Monaco et al., 1978; Monaco et al., 1980; Guillon,et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988). Vasopressin was shown to have a growth-promoting influence on MCF-7 breast cancer cells, presumably through V_1 vasopressin receptors (Taylor et al., 1990). Another study has demonstrated that vasopressin-induces a rise in intracellular free calcium in a human breast cancer cell line (Bunn et al., 1992). In a transgenic mouse model of breast cancer, ectopic vasopressin was found to stimulate cancer growth, but did not influence the time to tumor onset (Chooi et al., 1994).

Using antibodies directed against various regions of the vasopressin prohormone and the technique of immunohistochemistry we found that expression of vasopressin gene-related products is a common feature of breast cancers (North et al., 1995). In the present study it was found that the various cases of fibrocystic disease did not exhibit staining with antibodies directed against vasopressin or vasopressin-associated glycopeptide. These two antibodies had previously given positive immunostaining with all of the breast cancer specimens examined. The results obtained in the present study suggest that expression of vasopressin gene-related products is associated with the carcinogenic process and not with benign breast disease. These results also indicate that expression of vasopressin gene-related products is not just a marker of proliferation in the breast as indicated by the lack of staining of the tissue sections consisting of hyperplasia. Examination of subsequent medical records for these patients indicated that 3 of these cases (1 case of fibrocystic disease without hyperplasia, 1 case of fibrocystic disease with

hyperplasia, 1 case of fibrocystic disease with atypical hyperplasia) went on to develop breast cancer. Although the sample number is small these findings suggest that vasopressin gene-related products are not markers of premalignant lesions which will subsequently develop into breast cancer. Such findings are consistent with those obtained by Chooi et al. (1994) with MMTV-VP vasopressin transgenic mice. In these animals it was observed that vasopressin had no influence on normal mammary gland function and development, and did not cause the development of hyperplastic alveolar nodules and ductal hyperplasia. Taken together, these data are suggestive that vasopressin gene expression is not involved in benign breast disease, and is not a marker of preneoplastic changes in the breast. It would therefore seem to be a marker of the carcinogenic process in the breast. We are in the process of examining cases of carcinoma *in situ* to determine if vasopressin gene-related products are detectable at this stage of breast cancer.

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Figure 1 **a,** Positive staining for vasopressin-associated glycopeptide in a tissue section of infiltrating ductal breast cancer. Note the lack of staining in a normal structure, as indicated by the arrow (magnification = 147 x). **b,** Negative staining for vasopressin in a tissue section of fibrocystic disease with atypical ductal hyperplasia (magnification = 147 x). **c,** Negative staining for vasopressin-associated glycopeptide in a tissue section of fibrocystic disease with adenosis (magnification = 147 x).

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ELSEVIER

Ionic Signals in T47D Human Breast Cancer Cells

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ABSTRACT. Increasing evidence that ion channels play a key role in the modulation of cellular mitogenesis led us to investigate the membranes of T47D human breast cancer cells to identify the ion currents present. We report here the results of voltage-clamp studies in the whole-cell configuration on isolated, non-synchronized single cells obtained from a ductal breast carcinoma. In these studies we identified an outward rectifying potassium current and a chloride current. The potassium current activated at potentials more positive than −40 mV, reached an average value of 1.4 nA, and did not inactivate with time. This current was sensitive to block by extracellular tetraethylammonium chloride (TEA, IC_{50} = 1 μM), was insensitive to charybdotoxin (CTX, IC_{50} = 7.8 μM), and was not diminished by repetitive pulses separated by 1 s. Rapid voltage-dependent inactivation of the current was demonstrated by tail current analysis. The current appeared calcium-insensitive. Application of hyperpolarizing pulses did not elicit an inward potassium rectifier current. Treatment with tetrodotoxin did not reveal the presence of an inward sodium current. The potassium current was increased by the presence of aspartate in place of chloride and in the presence of the chloride channel blocker 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). We conclude that currents present in T47D breast cancer cells include a chloride current and a voltage-gated potassium outward rectifier. We suggest that the potassium current, either alone or in conjunction with potassium currents reported in different human breast cancer cell lines by others, may play a role in the modulation of the cell cycle. CELL SIGNAL 8;4:279–284, 1996.

KEY WORDS. Ion channels, Breast cancer, Potassium currents, Mitogenic signals

INTRODUCTION

An increasing body of evidence supports the hypothesis that potassium currents signal cellular proliferation [1]. Small-cell lung carcinoma cells (SCCL) treated with the K^+-channel antagonist 4-aminopyridine (4-AP) demonstrate an attenuated outward K^+ current coincident with a decrease in cell proliferation [2]. In MCF-7 breast cancer cells the potassium channel antagonists quinidine, glibenclamide, and linoglíride inhibit cell proliferation and cause the accumulation of cells in the G_2/G_1 phase of the cell cycle [3]. This effect on MCF-7 cells was attributed to the putative activity of adenosine triphosphate (ATP)-sensitive potassium channels. This cell line, established from pleural fluid of a patient with adenocarcinoma, retains certain characteristics of differentiated epithelium, including the presence of estrogen receptors [4]. A calcium-activated potassium current has been identified in MCF-7 cells [5] that, although correlated with cell proliferation, was not considered obligatory for growth [6].

The T47D breast cancer cell line selected for this study, originally established from the pleural effusion of a patient with infiltrating ductal carcinoma [7], also displays characteristics of an epithelial origin. These cells possess receptors for estrogen [8, 9], progesterone [6, 10–12], calcitonin [13], and vitamin D [14, 15]. We selected this cell line for investigation because estrogen-resistant clones have been produced, and we aim to develop this line as a model for evaluating breast cancer progression [16–18]. In view of the previous studies that have linked ion channels with mitogenesis, we initiated this study to identify the whole-cell currents present in unsynchronized cells of the T47D human breast cancer cell line.

MATERIALS AND METHODS

Cell Culture

The T47D human breast ductal carcinoma cell line was obtained from the American Type Culture Collection (ATCC HTB 133, Rockville, MD), and maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 0.2 IU bovine insulin/ml (Sigma, St. Louis, MO) and 10%

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fetal bovine serum (FBS; Hyclone Labs, Logan, UT). Cells received fresh growth medium or were subcultured (1:3 using 0.25% trypsin + 0.02% ethylenediamine tetraacetic acid [EDTA]) every 2–3 d.

Cell Preparation

Cells were plated onto glass coverslips approximately 18 h before patch-clamp studies were initiated. Each coverslip was placed in a chamber of 0.2 mL volume and washed with extracellular physiological solution. The chamber was placed on the stage of an inverted Nikon microscope and viewed with Hoffman optics (×640). Electrode preparation and other experimental details have been presented previously [19–21].

Solutions

The physiological external bath solution for recording whole-cell currents contained the following (in mM): 140 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 10 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethanesulfonic acid (HEPES), pH balanced to 7.3 with NaOH. The pipette solution contained (in mM) 140 KCl, 1.1 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 $CaCl_2$, 2.0 $MgCl_2$, and 10 HEPES, pH balanced to 7.3 with KOH. [Ionized Ca^{2+}] = 10^{-8} M. All experiments were done at room temperature (22°C). Extracellular KCl was increased by substituting KCl for NaCl in the bath solution. Extracellular Cl was reduced by substituting potassium aspartate for KCl in the bath. Intracellular [Ca^{2+}] was increased by changing pipette solution $CaCl_2$ to 1.08 mM [free Ca^{2+}] = 4×10^{-6} M.

Blocking Agents

Tetraethylammonium chloride (TEA; Sigma) was dissolved in extracellular solution and added in different concentrations directly to the bath. Charybdotoxin (CTX) from *Leiurus* venom was obtained from Alomone Labs (Jerusalem, Israel). The CTX was dissolved in 0.1% BSA, 100 mM NaCl, 10 mM Tris (pH 7.5) and 1 mM EDTA. This solution required the addition of 0.01% bovine serum albumin (BSA) to the bath. Because the bath total volume was 0.2 mL, replacement of the bath required only two drops of fluid. Replacement of the bath effected rapid and complete mixing of experimental solutions, as demonstrated by addition of a water-soluble dye. 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a recognized Cl^--channel blocking agent, was added to the bath.

Data Acquisition and Analysis

Currents in the individual cells were measured by rupturing the cell membrane in the lumen of the patch electrode by suction. Patch-clamp data were obtained with a List-EPC 7 clamp circuit (Medical Systems, Greenvale, NY). Experimental protocols, details of which are presented in the figure legends, were written for PCLAMP software (Axon Instruments, Burlingame, CA). Data were digitized at 5 KHz and recorded with a 486 PC computer after filtering with an 8 pole Bessel filter at 1 KHz. Series resistance correction, capacity compensation, and leakage subtraction were done. All data are reported as mean ± S.E.M.

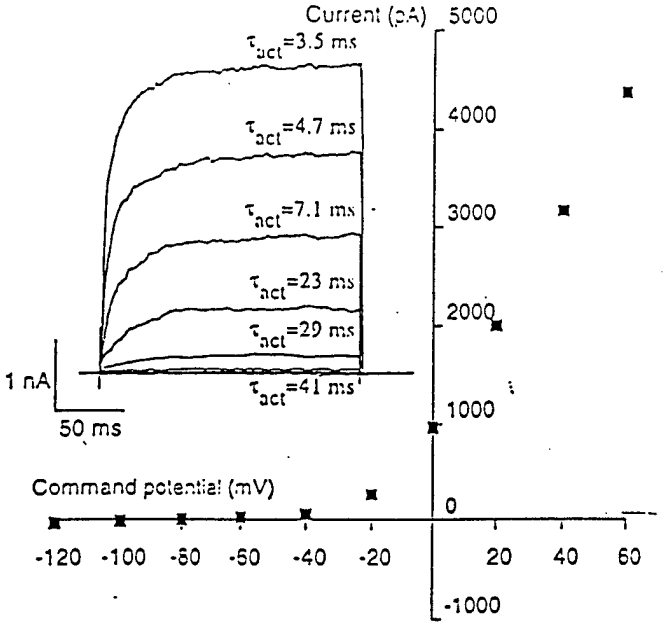

FIGURE 1. Outward rectifying K^+ current in T47D breast cancer cells. Current *vs.* voltage curve showing peak outward currents recorded in a cell held at −80 mV and then stepped to voltages from −120 mV to +60 mV. Inset: Currents recorded from this cell. Note the lack of time-dependent inactivation. The time constant for activation (τ_{act}) was best described by a single exponential for voltage steps to −40, −20, and 0 mV, and by a double exponential at more positive depolarizations. The fast time constant is shown for each trace.

RESULTS

Patch-clamp recordings and analyses of T47D human breast cancer cells revealed the presence of a voltage-sensitive current. This current was identified as a voltage-gated, time-independent, TEA-sensitive, charybdotoxin-insensitive, outwardly rectifying potassium current, and was present in 71 of 79 cells studied. We found no evidence of an inward rectifying potassium current or of a sodium current.

Voltage-gated Potassium Current

After rupture of the cell membrane by suction, voltage steps were applied to the cell in a normal physiological ionic gradient from a holding potential of −80 mV to command potentials of −120 mV to 60 mV. The large outward currents elicited are shown in the inset of Figure 1. The current was activated at −39.7 ± 2.2 mV (n = 10) and showed no time-dependent inactivation. At +40 mV depolarizations, the current reached a peak value of 1,376 ± 231 pA (n = 20). The time constants for activation could be represented by a single exponential at more negative voltage levels and

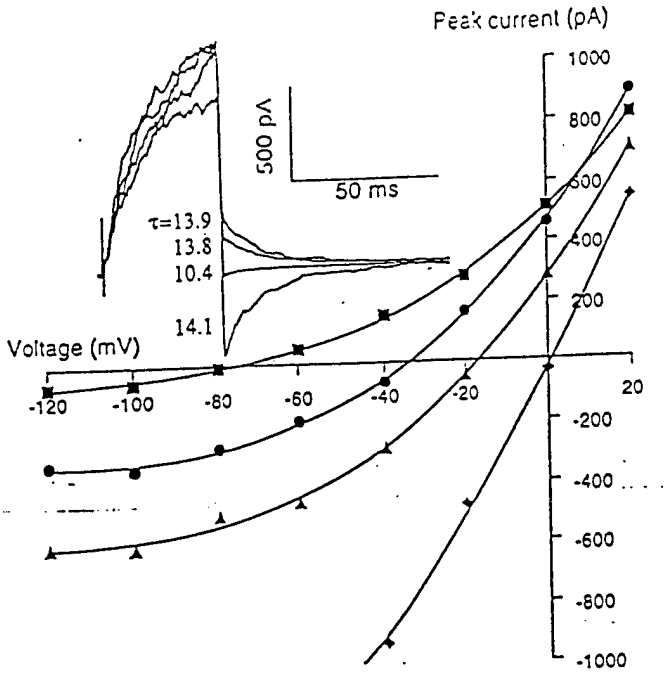

FIGURE 2. Tail currents. Peak current *vs.* voltage curves for tail currents recorded after a step depolarization from −80 mV to +40 mV. Pipette KCl concentration = 140 mM. External KCl concentration = 4.5 mM (squares), 38 mM (circles), 72 mM (triangles), or 140 mM (diamonds). Inset: Tail currents recorded at −20 mV in each external KCl concentration, with single exponential time constants (ms).

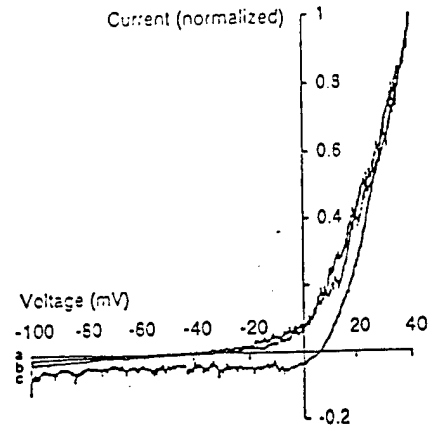

FIGURE 3. Voltage ramps in T47D breast cancer cells. From a holding potential of −80 mV, voltage ramps from −100 to +40 mV are shown. Curve a shows standard solutions. In curve b, pipette Ca^{2+} concentration was increased. The similarity between curves a and b suggests that no calcium-activated currents were present. Increasing external KCl concentration to 140 mM (c) alters the current *vs.* voltage relationships as expected. No evidence of an inwardly rectifying K current is seen, although the increase in leakage current suggests the presence of other non-voltage-activated currents.

by a double exponential at more positive depolarizations. The fast time constants for activation are indicated for each current trace.

Tail Current Analyses

The outward current was activated by a voltage step from a holding potential of −80 mV to +40 mV. V_m was then stepped to voltages between +20 and −120 mV and tail currents were recorded as shown in Figure 2. Rapid voltage-dependent inactivation was observed. Changing bath KCl concentration produced changes in reversal potential for the outward current. In external KCl concentrations of 4.5, 38, 72, and 140 mM, E_{rev} was −84.5 ± 1.2 mV, −31.8 ± 1.7 mV, −17.6 ± 0.2 mV, and −2.6 ± 2.2 mV, respectively (n = 7). For these KCl concentrations, calculated E_{rev} for a K^+-selective current was −86.6 mV, −32.8 mV, −16.7 mV, and 0 mV, respectively. Thus, this current appears to be carried by K^+ ions. The rapid voltage-dependent recovery of the current is emphasized by the absence of use-dependent peak amplitude reduction when cells were repetitively depolarized at intervals of 1 s (n = 8).

Voltage Ramps

After holding the membrane potential at −80 mV, voltage ramps were applied to the cell membranes from −120 mV to +40 mV, with the resulting I *vs.* V graphs shown in Figure 3. These experiments were performed with standard pipette and bath solutions, and also after increasing pipette Ca^{2+} concentrations to levels as high as 4×10^{-6} and external KCl concentrations to 140 mM. In no case was a calcium-activated or inwardly rectifying current detected. However, the increased leakage current noted in symmetrical KCl solutions suggests the presence of additional K^+ currents, perhaps a ligand-modulated current or the ATP-sensitive current reported by Woodfork *et al.* [3].

TEA

Figure 4 illustrates the results of 5 experiments in which voltage steps from −80 mV (holding) to +20 mV were applied in the presence of increasing concentrations of TEA chloride. Exponential curve fitting allowed calculation of a 50% inhibitory concentration (IC_{50}) of 1 μM TEA chloride, indicating a marked sensitivity of the current to this substance.

CTX

Charybdotoxin, a toxic component isolated from the venom of the scorpion *Leiurus*, has been shown in a number of studies to selectively block calcium-activated K^+ channels and to have a strong blocking action on other types of K^+ channels. Results of 5 experiments in which cells were depolarized after exposure to CTX are shown in Figure 5. Calculated IC_{50} was 7.8 μM, demonstrating an insensitivity of the current to the blocking effects of CTX. Both the solvent and BSA were tested in the absence of CTX and were found to have no effect on currents. The solution containing CTX completely replaced the bath in order to assure complete mixing.

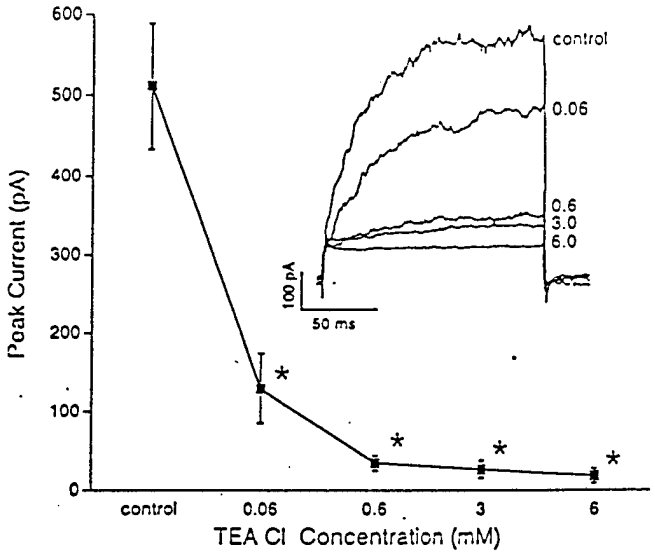

FIGURE 4. Effects of TEA chloride. Dose-related reduction in peak current following exposure to TEA chloride is demonstrated. Inset: Typical current recordings of a step depolarization from −80 mV to +20 mV after exposure to increasing TEA Cl concentrations. $^*P < 0.0001$ *vs.* control by one-way ANOVA for repeated measures and Tukey's Honestly Significant Different Test.

Inward Currents

SODIUM. To determine whether a sodium inward current was present, we added TTX (3×10^{-8} M) to the bath solution. The membrane was subjected to a more negative holding potential to remove any inactivation that might be present, and the membrane was then depolarized by step pulses. No effect on the whole-cell current was detected, leading us to conclude that a voltage-gated sodium current is not present.

POTASSIUM. Experiments were designed to test for the presence of an inward potassium rectifier. The membrane was stepped to various hyperpolarized potentials in physiological, reversed, and altered extracellular potassium concentrations. There were no currents detected under these conditions.

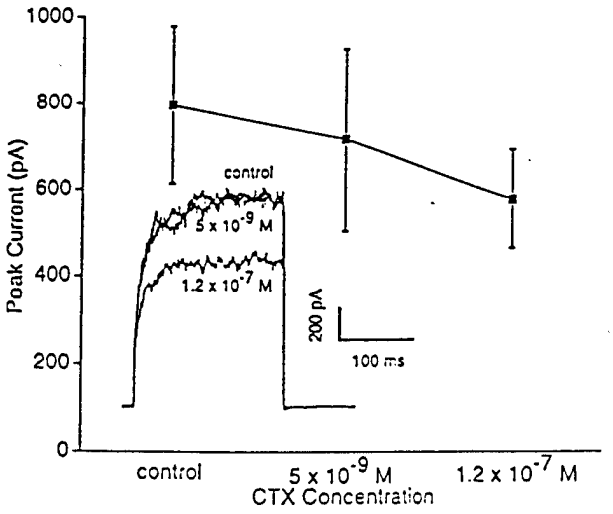

FIGURE 5. Effects of CTX. Increasing concentrations of CTX produce minimal decreases in peak outward current following a depolarization of −80 mV to +20 mV ($P = 0.11$). Inset: Typical current recordings after exposure to each concentration of CTX.

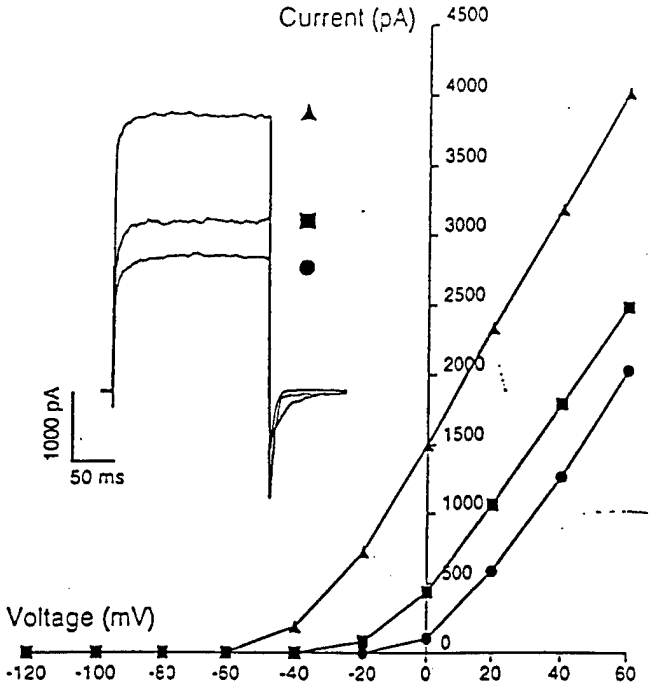

FIGURE 6. Augmentation of outward current by aspartate. From a holding potential of −80 mV, voltage steps from −120 to 60 mV were applied. The inset displays currents recorded after voltage steps to 60 mV in physiological bath solution (square), symmetrical 140 mM KCl solution (circle), and after substitution of 140 mM K aspartate for KCl (triangle). Current–voltage relationships for the same experiments demonstrate a marked increase in outward current after aspartate substitution.

CALCIUM. Increased amounts of calcium delivered both to the pipette and to the bath did not effect any changes in the outward current. With potassium replaced by CsCl in the pipette and with TEA in the bath, increased calcium in and out and addition of $BaCl_2$ (20 mM) did not reveal any inward current. Addition of CTX did not change the magnitude or time course of the potassium current. We conclude that calcium does not affect this voltage-gated potassium current.

CHLORIDE. The substitution of aspartate for chloride in the bath solution greatly enhanced the potassium current, as shown in Figure 6. The membrane potential was held at −80 mV, after which depolarizing steps were delivered from −120 mV to 60 mV. The marked increase in the total outward current is shown in the current traces and in the I *vs.* V relationship.

Chloride substitution experiments, tested by tail-current analyses, gave further evidence of the chloride component of the total current, as shown in Figure 7A–D. The cell membrane was held at −80 mV and then depolarized to 40 mV. Tail currents were then measured as the membrane

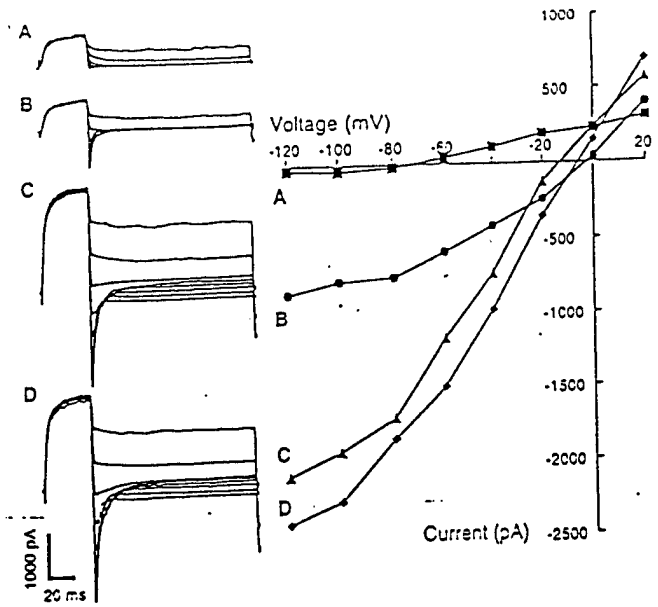

FIGURE 7. (A-D) Effects of chloride substitution on tail currents. Current traces A, B, C, and D display tail currents recorded after a depolarization to 40 mV from a holding potential of −80 mV. Shown are tail currents produced by voltage steps from 20 to −120 mV. The current-voltage curves for these traces are shown at the right. The pipette contained 140 mM KCl. Panel A was recorded in physiological bath solution. Panel B was recorded in symmetrical KCl solution and demonstrates a reversal potential near 0 mV. In C, 140 mM K aspartate was substituted for KCl in the bath solution and a change in reversal potential occurs. In D, 70 mM KCl with 70 mM K aspartate produced an intermediate change in reversal potential.

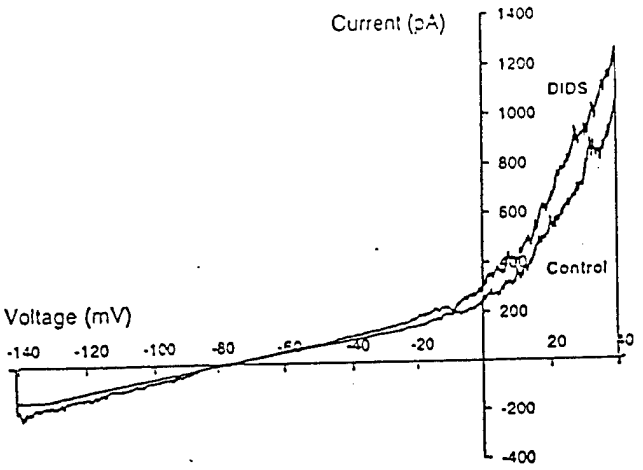

FIGURE 8. Increase in outward current after addition of DIDS. From a holding potential of −80 mV, a continuous ramp of increasing voltage from −140 mV to 40 mV was applied. The pipette contained 140 mM KCl. During the control recording, the bath contained standard physiological solution. After the addition of DIDS 4×10^{-4} M to the bath, peak current increased from 1,042 pA to 1,265 pA. This suggests that DIDS blocked an inward Cl^- current.

was stepped from 20 to −120 mV in a normal physiological gradient (Fig. 7A). The potassium content of the bathing solution was then elevated so that the potassium gradient was symmetrical (Fig. 7B). Under this condition, the reversal potential was near 0 mV, (I *vs.* V graph), as one would predict for a potassium current. The reversal potential was then observed to shift as K^+ aspartate was substituted for KCl (Fig. 7C). The reversal potential again shifted along the voltage axis as the bath was changed to contain 70 mM KCl and 70 mM K aspartate (Fig. 7D).

An increase in the outward current was also measured when the chloride channel blocker DIDS was added to the bath. Application of a continuous ramp of increasing voltage (−140 to 40 mV) from a holding voltage of −80 mV resulted in an increase in the outward current from 1,042 pA to 1,265 pA, as shown in Figure 8. We conclude from these data that a chloride current is present in these cells.

DISCUSSION

The voltage-gated potassium current we have characterized in T47D breast cancer cells activates rapidly at a membrane voltage of −40 mV, is outwardly rectifying, and displays no time-dependent inactivation. Peak currents are very large and at a depolarization level of 40 mV (holding: −80 mV), reached levels of 1.4 nA. This current is sensitive to block by TEA but not to CTX. TEA is a widely documented blocker of a potassium channels, but CTX is now known to block other potassium channels as well as the calcium-activated potassium channels [22]. In some cells, CTX blocks even the voltage-gated maxi-K channel [23]. In the experiments reported here, increased levels of internal calcium did not effect any change in either the magnitude of the potassium current or in the reversal potential, results that indicate that a calcium-activated potassium current is either not present or is of such relative magnitude compared to the outward rectifier that it is not readily visible by the whole-cell technique. In experiments in which the membrane holding potential was increased to hyperpolarized levels of −140 mV, no evidence of an inward rectifier was seen. The reversal potentials measured in a range of transmembrane potassium concentrations compared with the calculated values provide clear evidence for the ionic identity of the charge carrier. This current was not changed in the presence of tetrodotoxin, an observation that supports our conclusion that a sodium current is not present.

This report documents the first recordings of electrical activity from the T47D cell line of human breast cancer, an epithelial-like cell line obtained from an infiltrating ductal carcinoma of the breast. Another epithelial-like cell line of human breast cancer (MCF-7), obtained from an adenocarcinoma, has been explored by Woodfork *et al.* [3]. They concluded, from experiments using known potassium blockers on cell proliferation, that a ligand-activated potassium channel (i.e., an ATP-sensitive channel) is most likely involved in the proliferation of MCF-7 cells. In a preliminary report of voltage-clamp experiments [24], three types of current/voltage relations were described in MCF-7 cancer cells. In the presence of Mg-UDP (uridine diphosphate to enhance activation of K_{ATP}) in the pipette, linear I *vs.* V relationships

showed reversal potentials of −62 mV and −7 mV, respectively, while a third current was outwardly rectifying and reversed at −23 mV. Only the third type was observed when 2 mM ATP was also added to the pipette. The only current reported that appears similar to the outward rectifier we describe in T47D cells is a linear current with a reversal potential of −62 mV. The major potassium current in these two cell lines seems to be remarkably different. While we have not yet specifically studied the effects of ATP, we did note an increased leakage current in symmetrical potassium, which suggests the presence of an additional component.

Evidence is presented here for the presence in T47D cells of a chloride channel. The data include enhancement of the outward current by aspartate substitution for chloride, enhancement of the outward current in the presence of the chloride blocking agent DIDS, and the shift of the reversal potential in the presence of altered chloride concentrations in the bath. These data are consistent with the criteria for the identification of a chloride current.

In view of the complex nature and behavior of breast cancer cells, it is not difficult to appreciate that chloride ions may function in multiple roles (e.g., membrane stabilization, regulation of intracellular pH, modulation of secretion, and cell volume control). These cells exhibit prolific secretory behavior. Another possibility to be considered in regard to this chloride current is that the aspartate itself may unmask a different potassium component, since a variety of amino acids are known to exert excitatory effects on cells that have receptors for these agents [25]. Further studies should help to characterize and identify this component of the whole-cell currents.

Whether the apparent differences in the currents measured in the MCF7 and T47D cell lines are related to some basic property of these two types of cells or to variations in the stage of the cell cycle may be resolved in future studies using cell-cycle-synchronized cultures. Based on previous studies with other cell types [1], we suggest that it is more likely that the potassium current will be involved in modulation of the cell cycle while the chloride channel will be linked to secretion, regulation of cell volume, or some other cellular function.

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